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1. Your reference — 39471/HRW

2. Patent application number *(The Patent Office will fill in this part)* — 20 APR 1998 — 9808325.6

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

LTR C.I.Z. di
Associazione Italiana Allevatori
Via Porcellasco 7-f
26100 Cremona,
Italy

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of incorporation — Italian — 07419625001

4. Title of the invention

Source of Nuclei for Nuclear Transfer

5. Full name, address and postcode in the United Kingdom to which all correspondence relating to this form and translation should be sent

Reddie & Grose
16 Theobalds Road
LONDON
WC1X 8PL

91001

Patents ADP number *(if you know it)*

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application *(If you know it)*	Date of filing *(day/month/year)*

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Number of earlier application	Date of filing *(day/month/year)*

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a) any applicant named in part 3 is not an inventor, or
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Description	13
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11. I/We request the grant of a patent on the basis of this application.

Signature: Roddie & Grose

Date: 20 April 1998

12. Name and daytime telephone number of person to contact in the United Kingdom: H R WAKERLEY 0171-242 0901

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Source of nuclei for nuclear transfer

This invention relates to the generation of animals genetically identical to an existing or existed animal. Further, during the process of regeneration some characteristic(s) can be changed by ~~recombinant DNA technology to produce a transgenic animal by the~~ addition or deletion of selected genes.

Known procedures for nuclear transfer involve the transfer of a nucleus taken from a pre-implantation stage embryo into an enucleated mature oocyte. Following activation of the oocyte, in a process that mimics sperm entry and signalling, an embryo develops and eventually an individual that is genetically (as far as DNA is concerned) identical to the donor embryo. The limited number of cells present in a mammalian pre-implantation embryo, however, allows the regeneration of a limited number of embryos. Pre-implantation embryo nuclei donors do not allow the use of recombinant DNA technology because of the limited number of cells available. Most importantly, though, the genetic value of the embryo, and thus of the animal that will be born, can only be estimated. This is of low economic value. For these reasons the potential of nucleus transfer technology has not been developed with commercial exploitation in mind; its major use is for scientific purposes.

A partial solution to the limited number of nuclei has been the use of a so called 'serial nucleus transfer' where the embryos obtained from the starting embryos are further subjected once, or more than once, to the same procedure therefore increasing the number of embryos regenerated (Stice & al., 1991, Theriogenology 35, 273).

The major limitations to the use of nucleus transfer procedure outlined above would be overcome if a renewable and / or unlimited

source of nuclei to be used in the process could be made available. For many years people have attempted to establish cell lines from pre-implantation embryos (embryonic stem cell lines) but failed except for the mouse. Such work is reviewed in Galli et al. 1994, Zygote 2: 385-389. This type of cell would represent the ideal source of nuclei, however in the mouse they have never been used in nucleus transfer experiments.

Cultured inner cell mass cells or presumptive embryonic cell lines have been obtained and successfully used for nucleus transfer experiments to produce embryos: Moor, Sun & Galli, 1992, Animal Reprod. Sci. 28, 423-431; Stice & al. 1996, Biol. Reprod. 54, 100-110. Viable offspring have been produced in cattle and sheep: Sims & First, 1994, Proc. Natl. Acad. Sci. USA 91: 6143-6147; Campbell & al. 1996, Nature 380, 64-66; Wells & al. 1997, Biol. Reprod. 57,385-393. More recently, viable offspring has also been obtained with the use of nuclei from cultured fetal cells: Wilmut & at. 1997, Nature 385, 810-813; see The New York Times 21 January 1998 and Nature 392, 113, 1998. One lamb has been produced from a sample taken from a primary culture containing mainly mammary epithelial cells of an adult sheep: Wilmut & at. 1997, Nature 385, 810-813.

The advantages of using a renewable source of cell or a cell line in nucleus transfer procedure are:
- cells can be easily collected, cultivated and / or stored in liquid nitrogen;
- an unlimited number of embryos could be produced over a long period;
- cells can readily be modified in vitro using recombinant DNA technology.

There has been discussion about using nuclei of somatic cells collected from adult animals. This will have particular application

for livestock species where the value of an animal is determined by his progeny if a sire or by her production records if, for example, a dam. To regenerate a unique animal for production or genetic characteristics (transgenic) it is imperative to use nuclei from an animal which is an adult or one which has at least been born alive. That is not the case for the work using fetal or embryonic cells as a source of nuclei.

Description of the invention

The present invention provides a method of reconstructing an animal, preferably mammalian, embryo, the method comprising transferring a cell from hematopoietic lineage into a suitable recipient.

The cell from hematopoietic lineage can be transferred intact or the nucleus maybe extracted and used for transfer.

This invention finds particular application in the reconstruction of embryos of non-human animals, preferably those of mammals, and most preferably ungulate species embryos. Preferably the cell from hemtapoietic lineage is collected from an adult animal or an animal from a viable birth.

The invention further provides a method of reconstructing an animal, preferably mammalian, embryo comprising reconstructing a first generation embryo by the steps of a method according to the first aspect of the invention and then transferring a cell from the said first generation embryo to a suitable recipient to form a second generation embryo.

The invention still further provides a method of preparing an animal, the method comprising reconstructing an animal, preferably mammalian, embryo using a method described above; allowing the embryo so produced to develop to term; and, optionally, breeding from the animal so formed.

The present invention further provides a method of preparing embryonic stem cell lines, comprising reconstructing an animal, preferably mammalian embryo using a method described above; and transferring the embryo to a culture system.

The present invention further provides a method of preparing embryonic stem cell lines, comprising reconstructing an animal, preferably mammalian embryo using a method described above; isolating the inner cell mass of the embryo from the embryo; and transferring the inner cell mass to a culture system.

The culture system allows the embryo cells to attach, outgrow and produce a cell line with embryonic characteristics.

The present invention utilises cell from hematopoietic lineage and their derivatives partially or completely differentiated. These are mononuclear cells of hematopoietic lineage. These cells are present in bone marrow, lymphoid organs and in peripheral blood. They are also found in the umbilical cord of new-born animals. The intact cells or their isolated nuclei are used as a source of nuclei

in conventional nucleus transfer procedures. White blood cells can be collected from circulating blood, bone marrow, cord blood or lymphoid organs. It can be enriched and purified by means of density gradient centrifugation or other means of separation, like immunomagnetic separation, column filtration or similar techniques. ~~Mononuclear cells contain different lineages and their~~ undifferentiated precursors and it is not known if any mononuclear cell is suitable for this invention or if it is a sub population of differentiated or partially differentiated cells or uncommitted progenitors that can be used successfully.

Mononuclear cells derive from the hematopoietic stem cell that is present in the bone marrow, more committed progenitors of the myeloid and lymphoid lineage are also present in the circulating blood where they divide and differentiate in the different blood cells. These cells are characterised by immunocytochemestry and do not express cytokeratins as well as lamin A/C that are typical of differentiated cells: Galli & al. 1995, Proc. of the Italian Soc. of Vet. Sci. XLIX, 303-304; Rober, RA & al., 1990, J. Cell Sci. 95, 587-598. To this extent, the hematopoietic lineage shares some characteristics with embryonic cells that are also negative for cytokeratins and lamin A/C: Galli et al. 1994, Zygote 2: 385-389. This could explain in part the successful reprogramming of these nuclei into the cytoplasm of enucleated matured oocytes.

Freshly collected mononuclear cells can be cultured in vitro and are karyotypically normal. This latter characteristic is a prerequisite for the normal development of any individual, but it is not guaranteed by other cell types that have to be cultured for a length of time and where a degree of aneuploidy always occurs. Mononuclear cells can also be cultured in vitro for a time sufficient to use recombinant DNA technology to alter their genetic constitution: Bordignon & al., 1995, Science 270, 470-475.

In principle this invention is applicable to all animals, but it will be useful in particular for livestock species such as cattle, buffaloes, sheep, goat, pigs, horses, rabbit and other species of economic relevance. It can also be used to preserve genetic material or to generate animals of endangered, exotic or rare ~~species. In humans, it could find beneficial application in its use~~ to generate embryonic stem cells from a patient as a source of compatible undifferentiated cells to be used in transplantation for the therapy of degenerative diseases.

After the reconstruction procedure whereby a nucleus of a mononuclear cell of hemtapoietic lineage is reprogrammed into the cytoplasm of an enucleated oocyte, there are several options for which this invention could be used. Mononuclear cells can be easily cryobanked and therefore offer an economic way of storing germplasm of animals. When the embryo is reconstructed it can be used not for reproduction but to generate undifferentiated embryonic cell lines to be used in cell therapy of the individual that donated them thus overcoming the problem of rejection. If the embryos obtained are used for the generation of an animal this can be done directly by transferring the pre-implantation embryos to a final recipient that will carry the embryo to term, or the embryo can be subjected to serial nucleus transfer and therefore generate further embryos in a process that is more efficient and probably will increase the chances of reprogramming the cell nucleus because is exposed to the egg's cytoplasm more than once in a short period.

The steps involved in the cloning of an animal using this invention are summarised:

Step 1 - isolate the donor cell required from circulating blood or other tissue; enrichment for the fraction of cells that is more efficient in the procedure; optionally the cells can be genetically

modified during a period of in vitro culture using recombinant DNA technology.

At this stage the cells can be cultured, cryopreserved following one of the established protocols for later use or used immediately for nucleus transfer.

Step 2 - maturation of the oocytes harvested from donor females at slaughter or from live donors and removal of the egg's metaphase plate to prepare the so called 'recipient cytoplast'.

Step 3 - transfer of the nucleus obtained in step 1 by direct micro-injection of the cell or of the isolated nucleus directly in the cytoplasm of the enucleated oocyte or by other means such as cell fusion that can be achieved using intact donor cells with chemical, electrical or viral means.

Established cell fusion methods include the use of fusion-promoting chemicals, such as polyethylene glycol; the use of a virus such as the Sendai virus; and electrical stimulation.

The oocyte is activated to mimic sperm entry and start the developmental programme of the oocyte. Activation may be by inducing calcium oscillations in the embryo by chemical (ionophore) or physical (electric current) means, following which the embryo is exposed to protein inhibitors that facilitate the exit from the metaphase arrest that is maintained upon new protein synthesis.

Step 4 - develop the reconstructed embryo to a stage where it can be transferred to the uterus of the final recipient or subject to a serial cloning procedure by disgregating the embryos obtained in single cells and restarting from step 2. Various known systems of culturing embryos can be used successfully.

The steps involved in the preparation of a stem cell line using this invention are summarised:

Obtain a preimplantation stage (morula or blastocyst) embryo following steps 1-4 described in the previous example.

Step 5 - Remove the zona pellucida of the embryo. Optionally, the inner cell mass may be isolated from the embryo, for example by mechanical means or by immunosurgery. Plate and culture the intact embryo or the isolated inner cell mass. Various known system of culturing embryonic stem cells may be used. The culture take place on a monolayer of fibroblasts and/or in defined media supplemented with the necessary growth factors (leukaemia inhibitor factor, stem cell factor and others) that are required to maintain the embryonic cell in an undifferentiated state.

Step 6 - Subculture using, for example, mechanical or enzyme dispersal of the embryonic cell outgrowths in new culture vessels to expand the number of cells until a stable cell line is obtained.

Step 7 - The cell line may be frozen for long term storage or the genetic constitution of the cells genetically modified using recombinant DNA technology.

Step 8 - Following genetic modification, the embryonic cell may be used in the cloning of a mammal by following steps 2 to 4 .

EXAMPLE

This is an example of the use of the invention for the cloning of a cattle but similarly it can be applied to other species and with cells of different hematopoietic origin.

Step 1 - Cell isolation

A blood sample was taken from a cow of proven genetic value by venipuncture with heparinized vacutainer. The blood was diluted 1:1 with phosphate buffer saline (PBS) and 7 ml were layered on 3 ml of a density gradient (Hystopaque density 1083 g/cm3, Sigma) and centrifuged at 1500g for 15-30 minutes, the mononuclear cells ~~stopping at the plasma Hystopaque interface. The 0.5 - 1 ml band of~~ mononuclear cells were recovered, transferred into a new centrifuge tube, further diluted with PBS and centrifuged again to wash the cells. This step was repeated once and the cells were finally resuspended in an appropriate culture medium.

Mononuclear cells were cryopreserved in medium supplemented with 10-20% serum and 10% DMSO (dimethyl sulfoxide) and packed for example in plastic straws (normally used to pack bovine semen), each containing convenient working aliquots of cells (0.5-2 million cells) required in each day the method of the invention was carried out.

Step 2 - Preparation of cytoplasts

Oocytes at the second metaphase were used. These oocytes were collected from ovaries of slaughtered animals or by ultrasound guided transvaginal recovery from live donors. After collection immature oocytes were subjected to a 15-20 hour maturation period until they reached the second metaphase, following protocols described by Galli & Lazzari, Anim. Reprod. Sci. 42, 371-379, 1996. Oocytes at the end of the maturation period were denuded from the surrounding follicle cells and stained with a fluorescent dye (Hoechst 33342) that stains the chromosomes in the metaphase plate. With the aid of a micromanipulator under an inverted microscope using a micropipette, the first polar body, with a small volume of cytoplasm surrounding it, was removed and checked under fluorescent light for the presence of the metaphase plate. After enucleation,

the cytoplasts obtained in this way were returned to culture.

Step 3 - Embryo reconstruction

Cells prepared in step 1 and cytoplasts prepared in step 2 were transferred to a manipulation chamber under an inverted microscope and each cytoplast was injected with a small micropipette with one cell as described in Tesarik & Mendoza, Human Reproduction 11, 772-779; 1996. It was important to make sure that the cytoplast membrane was broken and the cell or its nucleus was effectively injected in the cytoplasm ready to undergo the reprogramming events necessary to support embryonic development. After injection the cytoplasts were returned to culture for a period generally of 2-4 hours.

About 70-80% of the cytoplasts survived the injection procedure. At this stage the oocytes were activated by exposing sequentially the reconstructed embryos (cytoplasts) for 5-7 minutes to 5 μM of Ionomycin (Sigma) and then to 2.5 mM 6-DMAP (Dimethyl amino purine, Sigma) for 4-5 hours: Susko-Parrish & al. 1994, Dev. Biol. 166, 729-739. This mimics sperm entry and will start the developmental programme of the oocyte.

Step 4 - Embryo development

Following activation, the reconstructed embryos were transferred to an in vitro culture system generally used to develop fertilised oocytes to blastocysts. Embryos were cultured in microdrops of SOF (synthetic oviductal fluid, Gardner & al. 1994; Biol. Reprod, 50, 390-400) in an atmosphere of 5% CO_2, 5%O_2 in nitrogen at 38.5 °C.

A proportion of the embryos (5%) (see table 1) developed to the blastocyst stage and could therefore be transferred to synchronised

recipients or frozen for subsequent transfer.

With such embryos a pregnancy rate of about 50% was achieved, the most advanced stage obtained was a pregnancy aborted at 195 days of gestation (a normal bull calf of about 10 kg). Most of the pregnancies resulted in abortions between 60-120 days. At present there is an ongoing pregnancy which is 170 days old.

Second Generation Cloning

In the first generation cloning only 5% of the reconstructed embryos developed to the blastocyst stage - of those embryos that undergo normal development. By this, 16 cell stage by day 4 and compacted morula by day 6 are presumably those embryos in which the reprogramming of the introduced nucleus has occurred.

To increase the efficiency of the procedure the first generation products are subjected to a second generation cloning. This second generation cloning is more efficient because it uses blastomeres (16, 32, 64 cell stages) and also gives the DNA a second chance for reprogramming because it is recycled back into the cytoplast.

Embryos obtained in the first generation cloning were exposed to calcium and magnesium free HBSS (Hanks balanced salt solution) for 2-4 hours to separate the embryo into single isolated blastomeres.

Cytoplasts were prepared as described in step 2 and first activated (as described in step 3) before the blastomere nucleus was transferred. In this case, the intact blastomere was transferred to the perivitelline space of the cytoplast and electrofused. The fusion rate was usually high (in excess of 80%). Reconstructed embryos at this stage were transferred to the culture system described above. About 17% of the fused embryos develop to

blastocysts. At present, 10 such recloned embryos have been transferred and 4 pregnancies have been established (2 are 80 and 2 are 50 days old).

TABLE 1

Data referring to embryo production and fetal development of embryo reconstructed with injection of mononuclear cells

	total number	percentage
no of experiments	26	
no of oocytes injected	1923	
no of oocytes survived	1377	71.6
no of blastocysts	71	5.2
no of blastocysts transferred	23	
no of pregnancies at 42 days	14	60.9
no of abortions between 60-90 days	9	
no of abortions between 90-120 days	3	
no of abortions > 120 days	1	(195 days)
no of ongoing pregnancies	1	(170 days)

TABLE 2

Data referring to embryo production and fetal development of embryos produced by a second cloning of early stages (morula stage) embryos produced by microinjection of mononuclear cells.

	total number	percentage
no of experiments	6	
no of embryos reconstructed	395	
no of oocytes fused	369	76.4
no blastocysts	62	17.2
no of blastocysts transferred	12	
no of pregnancies at 42 days	4	

EFFECT OF THE INVENTION

The present invention provides a source of donor cells for nuclear transfer techniques which gives advantages over known donors. The use of cells from hematopoietic lineage makes for very easy sample collection, which can be from adult animals of known characteristics. The supply of donor cells is not limited. The donor cells can be readily modified in vitro using recombinant DNA technology.